REGIONAL LOCALIZATION OF NEURAL AND LENS ANTIGENS IN THE FROG EMBRYO IN RELATION TO INDUCTION

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A number of embryologists recently have attempted to characterize embryonic cells by their constituent proteins. This approach is of particular interest when this characterization is attempted before, or at the time of, embryonic determination since it might be expected that a protein, or proteins, usually associated with a given tissue would increase in amount once the differentiation and growth of that tissue has already begun. The serological experiments of Ebert *et al.* (1955) in localizing cardiac myosin and actin in the early chick blastoderm, and those of Ten Cate and Van Doorenmaalen (1950) in determining the time of appearance and location of the lens antigen in frog and chick embryos are examples of this approach.

In relation to embryonic induction it would appear to be of some theoretical significance to be able to map or localize the protein that may characterize the reacting tissue in an induction system. In the induction of the medullary plate by the underlying chorda mesoderm, where is the greater amount of neural antigen localized just before this induction occurs? Is there more specific neural protein in the inductor (chorda mesoderm) or in the reacting tissue (gastrula ectoderm)? If the inductor has more neural protein, then this may imply the passage of specific protein from the inducing to the reacting tissue and subsequent synthesis of this protein in the reacting tissue. If, on the other hand, more of the neural antigen is present in the gastrula ectoderm, this implies that the induction stimulus is of a less specific nature and may merely be an activator for the synthesis of more neural protein in the reacting tissue.

Materials and Methods

In order to examine this question, antisera were developed against adult male frog brains (*Rana pipiens*) and antisera developed against adult frog lenses and cattle lenses for a previous investigation (Flickinger *et al.*, 1955) were also utilized. The method of preparing the anti-lens sera was presented in the above paper. Two antisera against adult male frog brains were prepared by cutting open the brains and freeing them of all visible blood, washing them several times in cold 0.65% saline, homogenizing them in a glass tissue grinder in the cold, and injection of the supernate obtained from centrifugation at 3000 g. This supernate was about 1% protein as shown by nitrogen determinations. Intravenous injections of 0.5, 1.0 and 2.0 ml. and an intraperitoneal injection of 4.0 ml. were given on alternate days and constituted an injection series. Three such series of injections were administered a week apart with the modification that the whole uncentrifuged homogenate was injected intraperitoneally in the third series of injections. The rabbits were bled 7 days after completion of injections. One of the antisera

reacted with a 0.1% protein supernate from adults' brains at an antiserum dilution of 1/128. Obviously extracts of adult frog brains are not highly antigenic.

In the preparation of test antigens, early gastrulae (Shumway stage 10) were operated upon under sterile conditions and cut in four regions; ectoderm, dorsal mesoderm, ventral mesoderm and endoderm, as seen in Figure 1. Most of the large white yolky cells were removed from the ventral and dorsal mesoderm tissues. Recently hatched larvae (Shumway stage 19–20) were cut into three parts; head, trunk and gut (Fig. 1). The heads were removed by cutting verti-

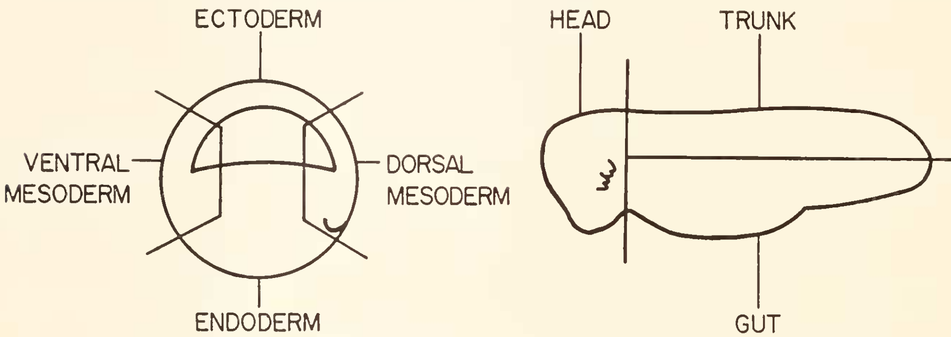

FIGURE 1. See text for explanation.

cally just posterior to the gill plate; the trunks were separated from the guts by cutting horizontally just ventral to the somites. No attempt was made to count the number of gastrulae and larvae that were operated upon, but for each preparation of test antigen from an embryo fraction, several hundred of the appropriate tissues were homogenized with an equal volume of 0.9% NaCl in micro-tissue grinders and then centrifuged in micro-centrifuge tubes (3 mm. inside diameter × 55 mm. length) in adaptors designed for the 7-ml. high speed head of the International centrifuge. After removal of the pigment, yolk, and lipid cap by repeated centrifugation at 15,000 g, some 0.2–0.3 ml. of a centrifugal supernate is obtained which can be used as the test antigen. The precipitin reactions were carried out in the same type of small tubes used for the centrifugations so as to conserve the antigen preparations. In some cases more concentrated embryo antigen preparations were obtained by homogenizing the embryo parts with an equal volume of supernate from a previous fractionation of the same tissues. A number of nitrogen determinations were made upon the test antigen preparations by the nesslerization method. The protein concentrations ranged from .35–1.5% protein, but for any given fractionation the protein concentrations were usually fairly similar for the four parts of the gastrula or the three regions of the larva. The test antigen preparations were adjusted to the same protein concentration before any given set of serological tests.

RESULTS

The anti-brain serum was not organ-specific and cross-reacted with frog serum, muscle, heart and kidney. It was found that serial absorption of 1.0 ml. of anti-brain serum with a total of 1.15 ml. of female frog serum, 1.0 ml. of a centrifugal supernate from a frog heart homogenate and 0.95 ml. of a frog kidney supernate

was sufficient to render the anti-brain serum specific. If the frog serum was not used in the absorptions then it required 2.4 ml. each of the heart supernate and the kidney supernate to render 1.0 ml. of the anti-brain serum organ specific. After absorption the antiserum did not react with centrifugal supernates obtained from adult frog heart, kidney, muscle, liver, spleen, ovary and frog serum.

In four separate experiments where the anti-adult male frog brain serum had been absorbed to completion with male frog serum, the antiserum showed a precipitin reaction with female frog serum. This was also found to hold true for similar absorption of an anti-adult male muscle serum. Upon further dilution with male frog serum the reactivity towards female frog serum disappeared. If the anti-brain serum was diluted 1/4 with normal rabbit serum and reacted against male and female frog sera, the latter reaction occurred at once while a faint precipitate did not appear in the male serum reaction for a period of twenty minutes. At the end of an hour the female serum-antiserum precipitate was much heavier than the male serum-antiserum reaction. It is known from agar-plate serology experiments of Flickinger and Rounds (1956) that an anti-embryonic yolk fraction serum gives 5 precipitate bands with female frog serum, and only two with male frog serum, so that the female frog serum apparently contains some proteins in higher concentration, or with different determinate groups, than those found in serum of the male frog. It is more difficult to understand the situation in this work where the antibodies have been formed in response to injections of an adult organ from a male frog. It might be expected that absorption to completion with male frog serum would not only remove activity to the male serum but also to female serum, especially since the serum contaminating the injection antigen was male serum. The most likely explanation is that the common frog serum proteins in male and female are in higher concentration in the female serum and therefore the female serum is a better antigen and can still react with anti-adult male organ sera absorbed to completion with male frog serum. This explanation seems more plausible than trying to invoke any type of pangenesis for adult organ antigens.

Anti-brain serum, rendered specific by absorption, reacted positively with all test antigen preparations from the four parts of the gastrula and the three regions of the larva (Fig. 1). The precipitates in every case were distinct after twenty minutes at room temperature; after twenty minutes at 37° C. and after twenty minutes at 6° C. the precipitates were still of equal intensity. Normal rabbit serum-antigen controls were negative. No attempt was made to titer the antiserum against the various test antigen fractions since it was apparent that the antigens reacting with the brain-specific antiserum were to be found in all regions of the gastrula and larva in approximately equal amounts. Absorption of previously unabsorbed anti-brain serum with the antigen fraction from the larval gut (where no elements of the nervous system are present) also removed activity against all other antigen fractions, thus indicating in another way that proteins bearing the determinate groups that react with the anti-adult brain serum are located in all regions of the frog gastrula and larva.

In order to see if different proteins may be given off by different tissues, as some of the work of Niu (1956) suggests, a series of forty explantation experiments was carried out in which 20–30 pieces of larval brain (stage 19) or larval trunk (Fig. 1) were stripped of their epidermis and cultured in the bottoms of deep well depression slides in Niu-Twitty solution (1953). After a week of explantation

the culture fluids were collected from above the tissues, centrifuged, and the supernates from the various cultures of denuded heads were pooled, as were the trunk culture supernates. There was some slight cytolysis in these cultures which would account for the release of some soluble protein into the medium, but it is felt that some of the protein probably was released into the medium by "natural means" other than cytolysis. These solutions were dialyzed against distilled water and then evaporated in dialysis bags suspended in front of a fan in a cold room (2–4° C.). An individual dialysis bag usually contained about 8 ml. of the culture supernate and this volume was reduced to about 0.2 ml. Nitrogen determinations in one case revealed the protein level of the concentrated supernate to be 0.06% protein. The absorbed brain-specific antiserum gave positive ring tests of equal intensity with both head and trunk supernates twenty minutes after the test antigens were layered over the antiserum. It would appear that the proteins given off from the denuded heads and trunks bear similar determinate groups that react with the brain-specific antibodies. These results are certainly not definite enough to state that proteins or nucleoproteins (inducing substances?) given off by different inductors are similar, even though much of the protein from the trunk cultures would be derived from the exposed myotomes and that from the head cultures would come from the brain, since the antiserum lacks the desired degree of specificity. However, the data tend to support the many biological examples of non-specificity of the inducing agent (Holtfreter, 1951).

The obvious disadvantage to the use of anti-brain serum is its lack of specificity. Hence it was decided to use anti-lens sera where it is known that this organ has a greater degree of organ specificity. This organ is of course also an induced organ and the localization of lens antigen in the embryo would be of value in relation to the induction problem. In a previous investigation (Flickinger *et al.*, 1955) anti-frog lens serum was used to demonstrate the presence of lens antigen in the anterior half, but not in the posterior half, of feeding frog larvae. However, the negative results do not necessarily mean an absence of the lens antigen but might imply only a reduced amount of the antigen in the posterior region of the embryo.

As a first attempt to improve the means of localizing lens antigen, flank ectoderm, which is known to possess the ability to respond to an induction stimulus and form lens, was stripped from several hundred hatched larvae (Shumway stage 19), homogenized in 0.9% NaCl, and a supernate fraction obtained by centrifugation. This extract did not react in the ring tests with an anti-cattle lens serum, or an antiserum to cattle lens α- crystalline (previously provided to me by Dr. Ten Cate of the University of Amsterdam). The anti-α-crystalline was also negative against the gastrula ectoderm and chorda mesoderm test antigens. It is believed that the epidermis alone did not provide sufficient soluble protein to give a precipitin reaction. Therefore, in order to concentrate the test antigen preparations, it was decided to use test antigens from the head and trunk regions of hatched larvae in which the tissues were homogenized with an equal volume of supernate of heads or trunks from a previous fractionation. This has an advantage over the use of posterior halves of larvae (Flickinger *et al.*, 1955) in that the less metabolically active gut region, containing more storage protein, is not included with the trunk tissues.

The anti-frog lens serum gave immediate strong ring tests with both the head

and trunk antigen preparations, but this antiserum also reacted with adult frog serum and therefore was not organ-specific. After absorption of 1 volume of anti-frog lens serum with ½ volume of female frog serum the antiserum was negative to serum, and still gave definite ring tests after 20 minutes with head and trunk supernates. This antiserum was negative against test antigen preparations from adult muscle, heart, kidney and ovarian supernate, but it did give a weak positive reaction with an adult brain supernate after one hour. Therefore three volumes of the anti-frog lens serum were absorbed with two volumes of a centrifugal supernate obtained from homogenizing adult frog brains with an equal volume of frog serum. This absorption left the anti-frog lens serum negative to serum and brain and the antiserum gave a reaction with the test antigen preparation from larval trunks.

The use of antiserum against adult frog lens in localizing the presence of lens protein, or a protein bearing lens determinate groups, has the disadvantage that the antibodies are directed against determinate groups characteristic of frog proteins as well as those characteristic of lens protein. It would be preferable to use an organ-specific anti-cattle lens serum where, if the antibodies did react with frog embryo test antigens, the reaction would most likely be due to the lens determinate groups of the frog antigens reacting with the antibodies directed against cattle lens proteins.

The anti-cattle lens serum was tested against frog serum, brain and kidney and found to give no reaction. However, this antiserum gave immediate strong positive reactions with the concentrated head and trunk antigens from the hatched larva. The appropriate normal rabbit serum-test antigen controls were negative. The anti-cattle lens serum did not react with test antigen preparations from the four parts of the early gastrula. This negative result might be explained by the fact the gastrula supernates were not prepared as concentrated antigens, as in the case of the larval heads and trunks, but it is also likely that the lens antigen is present in lower concentration at earlier stages.

In looking back at the previous work with this antiserum (Flickinger *et al.*, 1955) it was noted that the antiserum reacted with supernates from both the anterior and posterior halves of 69-hour chick embryos as well as with the ovarian supernate and hatching larva supernate from frog embryos.

Discussion

If antigens with neural determinate groups are localized in all parts of the gastrula and tailbud larva, and particularly in the more critical case where antigens with lens determinate groups are situated in the trunk region (somites, neural tube, notochord, and dorsal epidermis) of the early larva, this indicates that organ antigens may be more disperse for a certain time than the organ-forming areas in the embryos. Ebert *et al.*, (1955) have shown this to be the case for cardiac myosin in the chick blastoderm, although cardiac myosin did become localized in the heart-forming areas after a period of time. Cardiac actin was always confined to these heart-forming regions.

It seems that the presence of organ antigens outside their organ-forming districts does not invalidate the idea that one may in part characterize a cell, tissue or organ by the structural and functional (enzymes) proteins they contain. The wide distribution of organ-specific proteins is an indication of the totipotency of

various regions of the embryo which has been demonstrated by numerous transplantation experiments. It would also tend to support the idea that embryonic induction could be any one of a number of stimuli which might evoke protein synthesis at a particular region in the embryo.

For example: any specific protein, which the genetic machinery of the cells would allow to be synthesized, might be stimulated (induced) to this synthesis by a number of factors. Ribonucleic acid or ribonucleo-protein is a critical component for protein synthesis (Brachet, 1950; Gale and Folkes, 1954) and for embryonic induction (Niu and Twitty, 1953). It is known that protein synthesis is an endergonic process demanding energy (Fruton and Simmonds, 1953) and Miller (1939) has demonstrated the reversal of the anterior-posterior polarity of a section of stem of a regenerating hydroid by raising the temperature (and therefore the level of energy-yielding reactions) at the posterior end of the stem. The presence of free amino acids is known to be necessary for adaptive enzyme formation (Halvorson and Spiegelman, 1953) and it is known from the work of Barth (1941), Holtfreter (1945), Yamada (1950) and Flickinger (1958) that competent tissues can be stimulated to differentiate independently of an induction stimulus from another tissue by pH shock treatments which can dissolve yolk (Holtfreter, 1946). Flickinger (1957) has emphasized that the solubilization of yolk protein, which provides the material from which cytoplasmic proteins are synthesized, can be a causal step in embryonic induction. Even after specific cytoplasmic proteins have been synthesized the provision of an enzymatic substrate, as in Wilde's (1955) conversion of gastrula ectoderm cells into melanophores by giving them phenylalanine, could be considered an embryonic induction. Activators or inhibitors of an enzymatic reaction might then also act in an induction system. Viewed in this manner there may be multiple aspects of embryonic induction with any factor, or combination of factors, that would facilitate the synthesis, or activity, of specific proteins being considered an inductor. This is somewhat similar to the case shown by Spiegelman and Reiner (1947) where, under conditions optimal for growth and protein synthesis, adaptive enzymes may be formed without the substrate or inducer being present.

If a sub-differentiation threshold level of any given specific protein, or proteins, exists throughout the embryo, it may be that a preferential hierarchy of cell and tissue specialization exists such that when conditions become optimal for protein synthesis in a given region of the embryo then a specific cell type or tissue will be formed. That there is some kind of preferential hierarchy can be seen from the tendency for gastrula ectoderm activated by sub-lethal cytolysis to form forebrain structures (Holtfreter, 1944). This tendency to form head structures first can be seen in Sabella regeneration (Berrill, 1931) where the most anterior part forms first and then fills in the missing parts. From studies of regeneration and embryonic development it is evident that differentiation occurs time-wise along anterior-posterior and dorsal-ventral axes with the anterior and dorsal differentiations usually preceding the posterior and ventral ones. Possibly these anterior-posterior and dorsal-ventral patterns of specialization are due to gradients of factors which favor protein synthesis, and that certain types of cell or tissue specialization are favored when conditions for protein synthesis become optimal. For example, Flickinger (1957) has hypothesized that the primary organizer area forms where the first and most active conversion of yolk to cytoplasm occurs. The biological

totipotency of most parts of the embryo, and the serological evidence tend to indicate that some organ-specific proteins may be more widely distributed than the corresponding specific organ-forming areas. Possibly the postulated sequential protein synthesis, and cell specializations and growth that may depend upon these syntheses, are of a self-limiting type as postulated by Rose (1952, 1957) and Weiss (1952). The question why the synthesis of a given type of protein may be favored when conditions become optimal for protein synthesis is indeed puzzling. It is apparently not due to a purely qualitative distribution of protein, or to unequal nuclei (Briggs and King, 1952). It might be ascribed to a quantitative distribution of various organ-specific proteins, or nucleoproteins, but although there are gradient-wise distributions of soluble proteins and ribonucleoproteins, there is as yet no evidence concerning the specificity of these compounds. Another possibility might be a preferential sequence of activity of different specific genes.

It may be well at this time to review the idea of Driesch that the fate of a cell or tissue is a function of its position. It is well known that undetermined embryonic cells and tissues tend to "fit in" to the particular locale in which they find themselves. The fact that the nuclei of the cells of the determined neural plate are apparently undifferentiated and still able to promote complete development when injected into the enucleated egg (King and Briggs, 1954) and the apparent determination of the whole mouse embryonic shield before the determination of its individual constituent cells (Grobstein, 1952) argues for some sort of "supracellular patterning," perhaps of a polar or axial type (Child, 1941; Rose, 1957), which precedes cell specialization. This is a question which deserves a good deal of attention from embryologists.

Summary

1. Anti-adult male frog brain and muscle sera absorbed to completion with male frog serum still react with female frog serum. It is believed that serum proteins common to the male and female may be in higher concentration in the female serum and hence account for this reaction.

2. Anti-brain serum, rendered organ-specific by absorption, reacted positively with test antigen preparations from four regions of the early frog gastrula (ectoderm, dorsal mesoderm, ventral mesoderm, and endoderm) and three regions of the hatched frog larva (head, trunk and gut). The proteins bearing brain determinate groups are apparently situated throughout the embryo at these stages.

3. The organ-specific anti-brain serum gave positive precipitin reactions with culture supernates from both larval heads and trunks which had been denuded of their epidermis and explanted for a period of a week. The proteins given off by these cultured heads and trunks bear similar determinate groups that react with the brain antibodies.

4. An absorbed organ-specific anti-frog lens serum, and an organ-specific anti-cattle lens serum, reacted with concentrated test antigen preparations from both the heads and trunks of hatched frog larvae. It seems that lens antigen, or protein bearing lens determinate groups, is localized in areas other than the lens-forming region.

5. The significance of these results is discussed.

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